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Award Number: W81XWH-09-1-0303

TITLE: Development of a Nanotechnology Platform for Prostate Cancer Gene Therapy

PRINCIPAL INVESTIGATOR: Arash Hatefi (Ph.D.)

CONTRACTING ORGANIZATION:

Washington State University
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REPORT DATE: July 2010

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

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REPORT DOCUMENTATION PAGE

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1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-07-2010	Annual	1 JUL 2009 - 30 JUN 2010

4. TITLE AND SUBTITLE

Development of a Nanotechnology Platform for Prostate Cancer Gene Therapy

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-09-1-0303

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Arash Hatefi (PhD)

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Washington State University, Pullman, WA 99164-3140

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

X) Approved for public release; distribution unlimited
#

13. SUPPLEMENTARY NOTES

14. ABSTRACT: The objective of this research is to design and develop a nanocarrier that is able to evade the immune system, circulate in the blood stream, find its target prostate cancer cells, and transfer therapeutic genes into prostate cancer cells efficiently. The gene carrier is composed of: **a)** histone H2A peptide (H2A) to condense pDNA into nano-size particles (nanocarriers), **b)** a PC-3 specific targeting motif (TM) to target prostate cancer cells, **c)** an endosome disrupting motif (EDM) to disrupt endosome membrane and facilitate escape of the cargo into the cytosol, and **d)** a nuclear localization signal (NLS) to actively translocate pDNA towards the nucleus of cancer cells. The gene encoding the gene delivery system was synthesized and cloned into a pET21b vector. The vector was transformed into E.coli, expressed and purified. The purity (>96%) and expression of the gene delivery system was confirmed by SDS-PAGE and westernblot analysis. The gene carrier was complexed with plasmid DNA (pDNA) to form stable nanoparticles with sizes below 100nm. The nanoparticles were used to deliver reporter genes (pEGFP) to target PC-3 prostate cancer cells. The results demonstrated that the gene delivery system is able to target and efficiently transfect PC-3 cancer cells with minimum cross-reactivity with normal epithelial prostate cells. Furthermore, the gene delivery system by itself did not show any detectable toxicity in the range tested (110ug/ml).

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF: Unclassified			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
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Introduction

A major obstacle to improving patients' survival with advanced prostate cancer is progression of the cancer to androgen-independence. Therefore, methods such as gene therapy capable of delay or stop this progression may have a significant impact on improving patients' health. However, many challenges lie ahead for gene therapy, including improving DNA transfer efficiency to cancer cells, enhancing levels of gene expression, and overcoming immune responses. **The overall objective** of this research is to design and develop a nanocarrier that is able to evade the immune system, circulate in the blood stream, find its target prostate cancer cells, and transfer therapeutic genes into prostate cancer cells efficiently. The gene carrier is composed of: a) histone H2A peptide (H2A) to condense plasmid DNA (pDNA) into nano-size particles (nanocarriers), b) a PC-3 specific targeting motif (TM) to target prostate cancer cells, c) an endosome disrupting motif (EDM) to disrupt endosome membranes and facilitate escape of the cargo into the cytosol, and d) a nuclear localization signal (NLS) to actively translocate pDNA towards the nucleus of cancer cells. An elastin like polymer (ELP) has also been engineered in the vector structure to provide a hydrophilic shield and protect the vector/pDNA complex in the blood stream from the immune system. For simplicity, the vector will be shown as EDM-H2A-NLS-CS-TM or namely GHT2. PC-3 prostate cancer cells are selected as target because they are highly metastatic and characterized to be $CAR^{-}/HER2^{-}$. This means that they are not a good candidate for adenoviral gene therapy or Herceptin anti-HER2 immunotherapy. Therefore, development of a targeted delivery system for this type of prostate cancer cells could be highly beneficial.

CAR: Coxsackie Adenovirus Receptor

HER2: Human Epidermal Growth Factor Receptor 2

Body

For months 0-14, Task 1 was proposed. All the deadlines are met in time and we are currently several months ahead of schedule.

Task 1- Biosynthesize and characterize recombinant vectors composed of EDM, Histone H2A, NLS, CS, ELP and TM (months 0-14).

1.1. The genes encoding EDM-H2A-NLS-CS-ELP, EDM-H2A-NLS-CS-ELP-TM, and EDM-H2A-NLS-CS-TM will be synthesized and sequenced (months 0-2)

- The genes were designed and sent to IDTDNA Inc.(Coralville, IA) for synthesis. The genes were synthesized and the correctness of sequences was confirmed.

1.2. The genes will be cloned into pET21b vector using standard cloning techniques and sequenced (months 2-4)

- All genes were cloned into pET21b vector using standard cloning techniques and the sequences were verified.

1.3. The genes will be expressed and purified in *E. coli* expression system. The purity and expression levels will be confirmed by SDS-PAGE and westernblot analysis. The exact molecular weight and amino acid content will be determined by MALDI-TOF and amino acid content analysis (Common Wealth biotechnologies Inc., Richmond, VA). (months 4-10)

- All genes were successfully expressed in *E.coli* and purified to >96% purity. The SDS-PAGE and Westernblot analysis of the expressed EDM-H2A-NLS-CS-TM (GHT2) vector is shown in **Fig. 1** as an example. The expected molecular weight of the peptide is 23,631 daltons. A sample was sent to the Common Wealth Biotechnologies Inc. for amino acid content analysis. Unfortunately, due to the buffer intereference, no accurate measurements of the amino acid content could be performed. Nonetheless, the SDS-PAGE and westernblot analysis are sufficient to demonstrate the expression and purity of the vector.

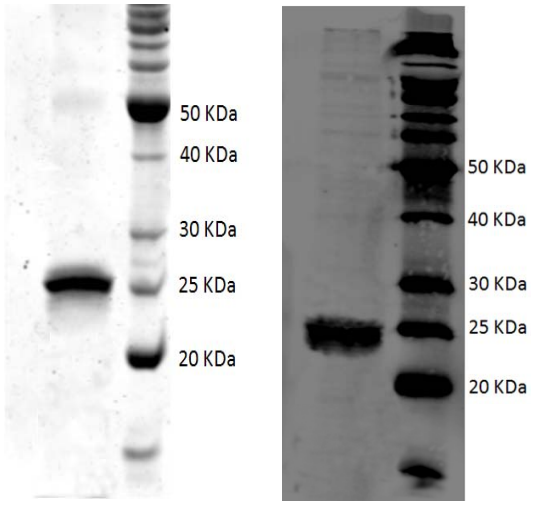

Fig. 1: SDS-PAGE (left panel) and westernblot (right panel) analysis of the purified EDM-H2A-NLS-CS-TM (GHT2) vector. This figures confirms the high purity and expression of the vector.

1.4. The expression will be optimized and scaled up in 6L culture medium using various culture media and under different growth conditions (months 10-13).

- Owing to the highly basic nature of the vectors and toxicity to *E.coli* system, the challenge had to be addressed by successfully screening a stable expression host (*E. coli*, BL21(DE3)pLysS) and optimizing the temperature, induction, extraction and purification conditions. Also, the storage conditions and the desalting techniques were major challenges which were resolved successfully. We currently have the complete protocol for the large scale production of the three vectors.

1.5. The ability of cathepsin D in cleaving its substrate will be examined and optimized. A cell toxicity assay will also be performed (months 13-14).

- The ability of the cathepsin D in cleaving its substrate was verified. The method used to make this observation has previously been published by our group [1].
- The results of the toxicity assay is presented below under task 2, section 2.2.

Task 2- Complex the vector with pDNA (i.e, pEGFP or pCM-Luc) to form nanocarriers and evaluate the transfection efficiency in vitro (months 14-24).

2.1. Vector/pDNA complexes will be formed and particles will be characterized under various physicochemical conditions including pH, temperature, and salt conditions. The nanoparticles will be stabilized and optimum conditions to form nanoparticles will be identified (months 14-17).

- The mean hydrodynamic particle size and charge of vector/pDNA complexes were determined using Dynamic Light Scattering (DLS) and Laser Doppler Velocimetry (LDV) respectively. All vectors were able to complex with pDNA and formed particles with sizes below 100nm. The particle size and charge analysis for GHT2 is shown in **Fig. 2.** This method has previously been published by our group [1]. The particles demonstrated stability under various pH, temperature (up to 37oC) and salt concentrations (up to 150mM NaCl). Because the nanoparticles demonstrated stability, they were used to transfect various cell lines.

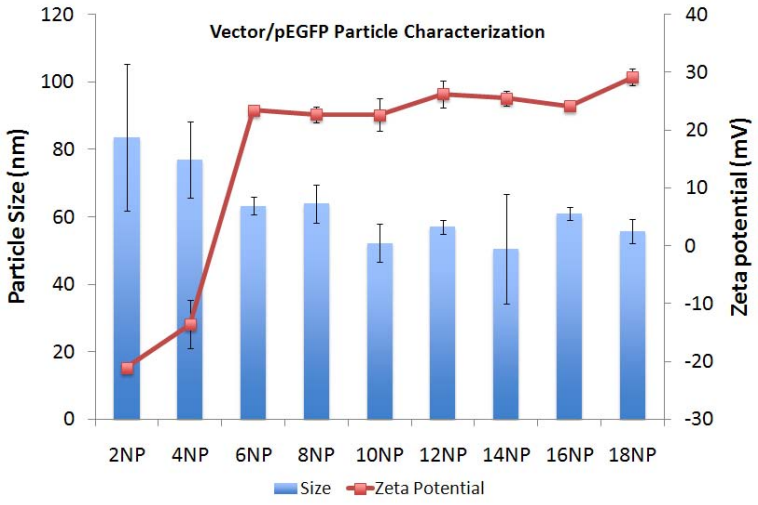

Fig. 2: Various amounts of vector in 5mM acetate buffer were added to 1 µg of pDNA (pEGFP) to form complexes at different N:P ratios (N-atoms in vector to P-atoms in pDNA) in a total volume of 100µl deionized water. After 30 minutes of incubation, the size and zeta potential of the complexes were measured and reported as mean ± SEM, (n=3). Each mean is the average of 15 measurements and n represents the number of separate batches prepared for the measurements.

2.2. Cell transfection studies will be performed using PC-3 and RWPE-2 cells. Cells will be transfected with vector/pEGFP complexes at various N/P ratios in the presence and absence of chloroquine, bafilomycin, and Nocodazole and the transfection efficiency will be measured. The cell transfection process will be optimized to obtain highest transfection efficiency (months 17-24)

- The transfection efficiency was optimized and the ability of the GHT2 vector to target and transfect PC-3 prostate cancer cells but not RWPE-1 normal epithelial prostate cells is shown in **Fig. 3**. The details of the method of cell transfection have been previously published by our group [1]. Please note that we had originally proposed to use RWPE-2 normal prostate epithelial cells. However, through consultation with Dr. G. Meadows (collaborator) who is a cancer biologist we replaced it with RWPE-1 which is a more appropriate cell line and a better representative of normal human prostate cells.

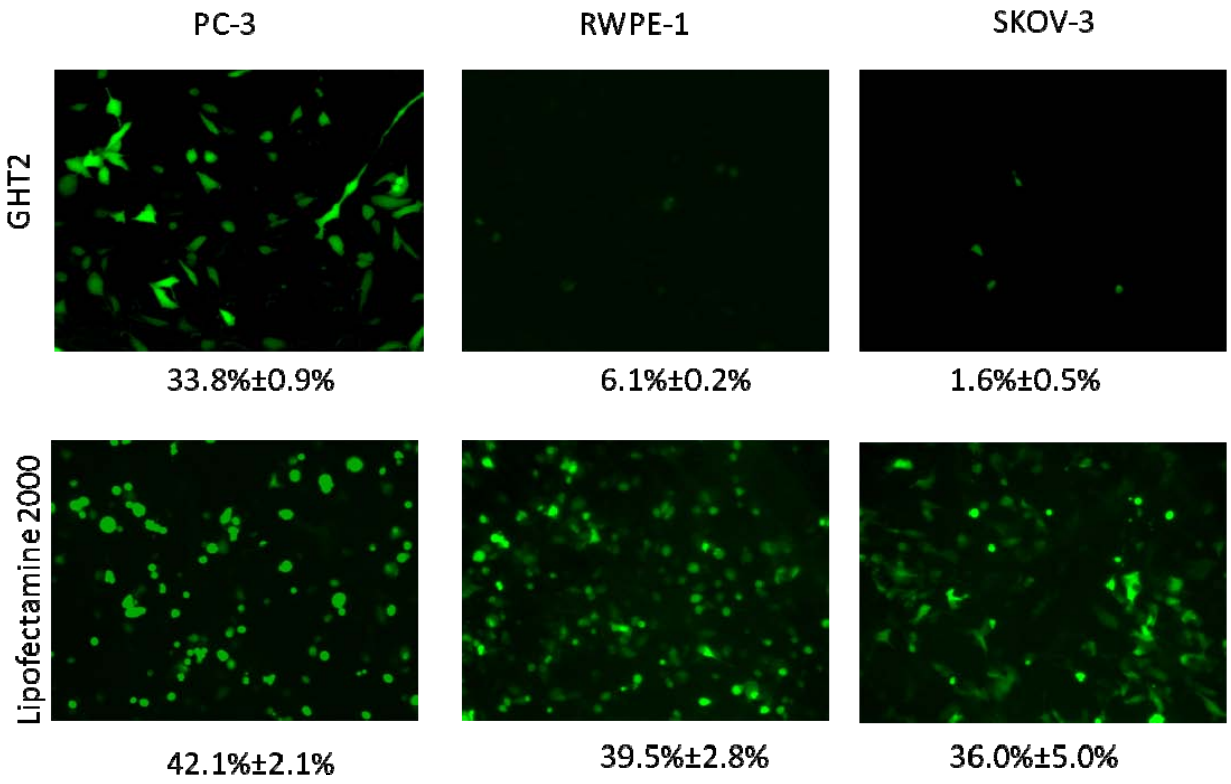

Fig. 3: Demonstration of PC-3 targeted gene delivery. Qualitative and quantitative representation of the PC-3 ($CAR^{-}/HER2^{-}$), RWPE-1 (normal cells) and SK-OV-3 cells ($CAR^{+}/HER2^{-}$) transfected with vector/pEGFP complexes at N:P ratio of 10 and lipofectamine/pEGFP. The percentage of transfected cells is measured by flowcytometer. This figure shows that the GHT vector can target and transfect prostate cancer cells while preserving normal cells. In contrast, commercially available vectors such as lipofectamine non-selectively transfect all cells which could result in unwanted toxicity to normal cells during the therapy.

- Using a WST-1 cell toxicity assay, the potential toxicity of the GHT2 vector to PC-3 cancer cells was evaluated. The details of this method have previously been published [1]. We did not observe any vector related toxicity in PC-3 cancer cells at any NP ratio tested **(Fig. 4).** At NP ratio of 18, 23.5µg of vector is used to complex with 1µg of pEGFP.

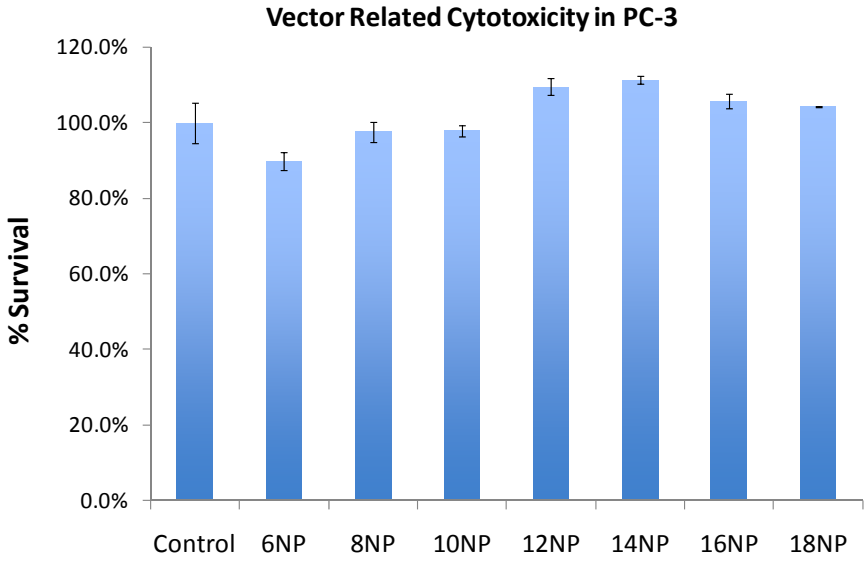

Fig. 4: PC-3 cells were seeded at 10,000 cells/well in 96-well plates. Cells were transfected with GHT2/pEGFP complexes formed at various N:P ratios (equivalent to 1ug pEGFP). The control group received no treatment. 48 hours after transfection, a WST-1 (Roche Applied Science, Indianapolis, IN) cytotoxicity assay was performed to determine the vector related cytotoxicity in this cell line.

Key Research Accomplishments

a) Developed reproducible expression and purification protocols for the production of highly basic vectors in E.coli system.
b) Engineered a vector composed of five independent functional domains.
c) The engineered vector demonstrated that it can target and transfect PC-3 prostate cancer cell specifically with high efficiency and with no detectable toxicity.

Reportable Outcome

A) Manuscript: In preparation

B) Presentations: The PI (A. Hatefi) has received an invitation from the organizers of the Nanomedicine and Drug Delivery Symposium (NanoDDS'10) to give a talk about this research. This symposium will be held on October 3-5, 2010. The data related to this research are expected

to be published in the Nanomedicine Edition of the Journal of Controlled Release. The Symposium Program Highlights which shows the name of the PI and the title of his talk is provided as an appendix (Appendix A).

C) Training: A Research Scientist with experience in molecular biology techniques was hired. She has received training in vector development, vector characterization, mammalian cell culture and transfection, and targeted prostate cancer gene therapy.

D) Grant application: None

E) Patent application: None

Conclusions

Using genetic engineering techniques we have created a PC-3 specific gene delivery system that can potentially be used in the treatment of the patients that do not respond to adenoviral gene therapy or Herceptin immunotherapy. The *in vivo* studies in nude mice are designed and will be performed in PI's new Institution (i.e., Department of Pharmaceutics at Rutgers).

References

[1]- Y. Wang, S.S. Mangipudi, B.F. Canine, **A. Hatefi**, A designer biomimetic vector with a chimeric architecture for targeted gene transfer. *J Control Release* 137 **(2009)** 46-53.

Appendices

Appendix A: The Nanomedicine and Drug Delivery Symposium (2010) program highlights.

nanomedicine and drug delivery symposium - omaha

October 3 - 5, 2010
Omaha, Nebraska

NanoDDS'10

Keynote Speakers

Joseph DeSimone (University of North Carolina at Chapel Hill)
Teruo Okano (Tokyo Women's Medical University)

Confirmed Speakers

Christine Allen (University of Toronto)
Mark Davis (California Institute of Technology)
Dennis Discher (University of Pennsylvania)
Iola Duarte (University of Aveiro)
Mohamed El-Sayed (University of Michigan)
Hamid Ghandehari (University of Utah)
Justin Hanes (The Johns Hopkins University)
Arash Hatefi (Washington State University)
W.E. Hennink (Utrecht University)
Leaf Huang (University of North Carolina at Chapel Hill)
Akihiro Kishimura (The University of Tokyo)
Philip S. Low (Purdue University)
Robert Luxenhofer (Dresden University of Technology)
Andrew Mackay (University of Southern California)
Muthiah Manoharan (Alnylam Pharmaceuticals)
Olivia Merkel (Philipps University, Marburg)
Xin Ming (University of North Carolina at Chapel Hill)
Tamara Minko (Rutgers University)
Randall Mrsny (University of Bath)
Vladimir Muzykantov (University of Pennsylvania)
Yukio Nagasaki (University of Tsukuba)
Derek O'Hagan (Novartis)
David Owen (Starpharma)
Robert Prud'homme (Princeton University)
Tom Redelmeier (Northern Lipids, Inc.)
Sonke Svenson (Cerulean Pharma Inc.)

Organizing Committee

Chair: Alexander Kabanov (University of Nebraska Medical Center)
Co-Chair: Tatiana Bronich (University of Nebraska Medical Center)
Christine Allen (University of Toronto)
Hamid Ghandehari (University of Utah)
Ralph Lipp (Eli Lilly & Company)
Christine Allmon (University of Nebraska Medical Center)
Marsha Fau (University of Nebraska Medical Center)
Keith Sutton (University of Nebraska Medical Center)

Registration and abstract submission: www.nanodds.org

nanomedicine and drug delivery symposium - NanoDDS'10 omaha

8th International Nanomedicine and Drug Delivery Symposium Omaha, NE, USA October 3-5, 2010

The field of nanomedicine has seen an exponential growth in the level of activity, investment, and development in recent years as evidenced by the creation of new companies, the launch of new scientific journals such as *Nature Nanotechnology*, *Nano Letters, and Nanomedicine-UK,* the establishment of new programs and departments in universities and government research institutions, as well as an extraordinary increase in the number of nanomedicine-focused publications. Developments in nanomedicine have already achieved true improvements in human health with the great promise of even more to come. Drugs relying on delivery or formulation in nanotechnologies such as Doxil™ have been approved for human use while others like NK911, NK105, and SP1049C have entered clinical trial development. Imaging agents for disease detection, characterization and staging such as Fenestra™ have also reached the late stages of pre-clinical development. While nanomedicine now features prominently in many scientific meetings, conferences and symposia, the NanoDDS meeting series remains unique among them.

NanoDDS, now entering its eighth year, began in 2003 as a US-Japan mini-symposium organized by Dr. A. Kabanov and Dr. K. Kataoka. According to Dr. Ruth Duncan it was the first scientific meeting of its kind. NanoDDS has since established itself as an internationally-recognized, medium-sized and focused symposium highlighting new discoveries and developments in the field of nanomedicine while also serving as a forum to discuss the issues of pre-clinical and clinical development of nanomedicines. The intimate format of NanoDDS sets it apart from all other meetings of its kind, providing an excellent environment for interaction, networking, discussion, creativity and innovation between trainees (graduate students and post-doctoral fellows) and some of the world's leading academic scientists and industry specialists. These interdisciplinary interactions should aid in fostering new, fruitful collaborations, leap-step advances, and ground-breaking discoveries.

SCIENTIFIC PROGRAM
Sunday October 3rd, 2010

First Keynote Presentation

Molecular Design of Intelligent Surfaces for Drug and Cell Delivery - *Teruo Okano, Tokyo Women's Medical University, Japan*

Session 1: Nanomedicines in Cancer (Part One)

Image Guided Design of Liposome-Based Cancer Therapy - *Christine Allen, Univ. of Toronto, Canada*

Ligand-Targeted Molecules for Imaging and Therapy of Cancer and Inflammatory Diseases
Philip S. Low,Purdue University

Nanotechnology Strategies to Overcome Limitations in Drug Delivery: Opportunities and Challenges - *Tamara Minko, Rutgers University*

Session 2: Nanomedicines in Cancer (Part Two)

Nanoparticle Delivery of siRNA for Cancer Therapy
Leaf Huang,University of North Carolina-Chapel Hill

Architectural Influence of Nanocarriers on Tumor Distribution and Toxicity
Hamid Ghandehari, University of Utah

From Concept to Clinic with Targeted Nanoparticles Containing siRNA
Mark E. Davis, California Institute of Technology

Session 3: Clinical Translation of Nanomedicines

Industrial Session and Roundtable - From Bench to Bedside: *Tom Redelmeier of Northern Lipids Inc., David Owen of Starpharma, Derek O'Hagan of Novartis, Sonke Svenson of Cerulean Pharma Inc., Muthiah Manoharan of Alnylam Pharmaceuticals*

Monday October 4th, 2010

Second Keynote Presentation

Engineering Better Medicines and Vaccines -*Joseph DeSimone, University of North Carolina-Chapel Hill*

Session Four: Novel Nanoformulation Technologies

Facile Production of Nanoparticles for Difficult to Deliver Therapeutics: hydrophobic drugs, peptides, and siRNA - *Robert Prud'homme, Princeton Univ.*

Novel Nanoparticles Functionalized for Anti-Oxidative Stress
Yukio Nagasaki, University of Tsukuba, Japan

Polymersomes to Filomicelles - thickness, shape, flexibility, charge...
Dennis Discher, University of Pennsylvania

Thermsensitive Polymeric Micelles for Tareted Delivery – *W. Hennink, Utrecht Univ., Netherlands*

Session Five: Tackling Nanomaterial–Tissue Interfaces to Improve Therapies and Diagnostics

Nanocarriers for Antioxidants
Vladimir Muzykantov, University of Pennsylvania

Strategies to Enhance Nanoparticle Transcytosis
Randall Mrsny, University of Bath, United Kingdom

The Mucus Barrier to Viral and Non-Viral Gene Therapy - *Justin Hanes, Johns Hopkins University*

Poster Sessions and Oral Presentations

Tuesday October 5th, 2010

Session 6: Nanomedicine Research Reports

Following Dynamic Biological Processes through NMR Spectroscopy and Metabonomic Profiling
Iola Duarte, University of Aveiro, Portugal

Customization of Genetically Engineered Vectors for Targeted Gene Transfer to Different Cancer Cells - *Arash Hatefi, Washington State University*

Novel polymeric hollow capsules "PICsomes"
Akihiro Kishimura, University of Tokyo, Japan

Doubly-Amphiphilic Poly(2-oxazoline)s with Unusual Microenvironment as High-Capacity Drug Delivery Systems - *Robert Luxenhofer, Dresden University of Technology, Germany*

Genetically Engineered Polypeptosomes
J. Andrew Mackay, Univ. of Southern California

SPECT Imaging of *in vivo* siRNA Delivery
Olivia Merkel, Philipps Univ.-Marburg, Germany

Molecular and Quantitative Pharmacology of siRNA Oligonucleotides Delivered via Receptor-Mediated Endocytosis - *Xin Ming, University of North Carolina at Chapel Hill*

Design and Evaluation of "Smart" Degradable Particles for Effective Gene Silencing
Mohamed E.H. El-Sayed, University of Michigan

GENERAL INFORMATION

Symposium Location

The symposium will be held at the Hilton Omaha, Nebraska's only 4 diamond hotel, located at 1001 Cass Street and within easy walking distance of the Old Market, Nebraska's premiere arts and entertainment district. The Hilton Omaha is only a short drive from Omaha's airport, as well as many other attractions including the Holland Performing Arts Center, Joslyn Museum, Durham Western Heritage Museum, and the Henry Doorly Zoo.

Accommodations

Accommodations are available at the Hilton Omaha at reduced conference rates. Alternate accommodations may also be found at several nearby hotels. Please visit the symposium website www.nanodds.org for additional information.

Symposium Registration

To register for the symposium, or for additional information, please visit the symposium website www.nanodds.org. Fees are $125 for graduate students and post-doctoral fellows, $400 for academics, and $1,200 for industry participants. These fees include admission to all three days of the symposium, an abstract book, name badge and conference packet, continental breakfasts, lunches, and refreshment breaks.

Call for Posters

The poster session will be the highlight of the symposium with time dedicated for poster viewing and discussion with the authors. A number of posters will be selected for oral presentations based on their scientific merit, innovation and clarity. Those interested in presenting their work during the poster session of the symposium should please visit www.nanodds.org for abstract submission guidelines and formats. Please email your abstract to nanodds@unmc.edu by Friday, August 27, 2010.

CONTACT

Questions about NanoDDS'10 may be directed to: nanodds@unmc.edu

www.nanodds.org